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13. ABSTRACT (Maximum 200 Words)

Mutational inactivation of BRCA1 confers a cumulative lifetime risk of breast and ovarian cancers. However, the underlying basis for the tissue-specific tumor suppressor properties of BRCA1 remains poorly defined. Previously, we described a novel function for BRCA1 in suppressing the ligand-independent transcriptional activity of the estrogen receptor α (ERα), a principal determinant of the growth and differentiation of breasts and ovaries. Based on these observations, we hypothesized that BRCA1 represents a ligand-reversible barrier to transcriptional activation by unliganded ERα and, further, that mutational inactivation of BRCA1 promotes breast epithelial cell proliferation through aberrant expression of estrogen-responsive genes, possibly contributing to tumorigenesis. To substantiate this hypothesis we have proposed the following aims: (1) to biochemically reconstitute BRCA1-mediated ligand-independent repression of ERα in vitro; (2) to examine the role of estrogen-induced site-specific BRCA1 phosphorylation in the regulation of BRCA1-mediated ligand-independent ERα repression; and (3) to determine the role of BRCA1 in the control of paracrine growth signaling in the breast. Collectively, these studies should reveal novel insight into the tissue-specific tumor suppressor function of BRCA1 and provide defined molecular targets for future intervention in breast cancer.

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INTRODUCTION

Mutational inactivation of the **BR**east **CA**ncer susceptibility gene product, BRCA1, confers a cumulative lifetime risk of breast and ovarian cancers (1,2). However, the underlying basis for the tissue-specific tumor suppressor properties of BRCA1 remains poorly defined. Previously, we described a novel function for BRCA1 in suppressing the ligand-independent transcriptional activity of the estrogen receptor α (ERα), a principal determinant of the growth and differentiation of breasts and ovaries (3; Please refer to Appendix 1– manuscript reprint). Importantly, we documented that clinically validated BRCA1 missense mutations abrogate this repression activity, thereby suggesting that its ERα-specific repression function is important for the biological activity of BRCA1 in breast and ovarian tumor suppression. In human breast cancer cells, we observed an association between BRCA1 and ERα at endogenous estrogen-responsive gene promoters before, but not after, estrogen stimulation. Furthermore, we demonstrated that attenuation of BRCA1 expression in estrogen-dependent human ovarian cancer cells could be correlated with increases in both the estrogen-independent transcription of ERα-target genes and estrogen-independent cellular proliferation. Based on these observations, we hypothesized that BRCA1 represents a ligand-reversible barrier to transcriptional activation by unliganded ERα and, further, that mutational inactivation of BRCA1 promotes breast epithelial cell proliferation through aberrant expression of estrogen-responsive genes, possibly contributing to tumorigenesis. To substantiate this hypothesis we proposed (1) to biochemically reconstitute BRCA1-mediated ligand-independent repression of ERα in vitro; (2) to examine the role of estrogen induced site-specific BRCA1 phosphorylation in the regulation of BRCA1-mediated ligand-independent ERα repression; and (3) to determine the role of BRCA1 in the control of paracrine growth signaling in the breast. These studies should reveal novel insight concerning how mutational inactivation of a ubiquitously expressed tumor suppressor could have restricted consequences in the breast and ovary. Furthermore, we expect these studies to have important implications with respect to the future treatment of breast cancer. Mechanistic insight into the biological role and regulation of BRCA1 as a repressor of ERα function should expedite the development of tissue-specific chemotherapeutic approaches intended to restore an appropriate hormonal response to BRCA1-mutant breast epithelial cells.

BODY

Technical Objective 1. To biochemically reconstitute BRCA1-mediated ligand-independent repression of estrogen receptor α (ERα) *in vitro* from purified components.

Task 1: ***Months 1-12:*** To reconstitute estrogen-independent ERα-directed transcriptional activation *in vitro*. This will be done using nuclear extracts derived from *Brca1-/-* mouse embryo fibroblasts (MEFs) and purified recombinantly expressed ERα on chromatin-assembled templates *in vitro*.

We have now successfully achieved the biochemical reconstitution of estrogen-independent ERα-directed transcriptional activation using nuclear extracts derived from *Brca1-/-* mouse embryo fibroblasts (MEFs) and highly purified recombinant ERα on an ERα-responsive reporter template *in vitro*. To this end, we first purified to near homogeneity human ERα bearing a FLAG epitope (fERα) following its overexpression in insect Sf21 cells using an M2 FLAG monoclonal antibody affinity column (Fig. 1). fERα thus purified was then tested for its ability to support activated transcription in *Brca1-/-* MEF nuclear extract from a reporter template bearing four copies of the

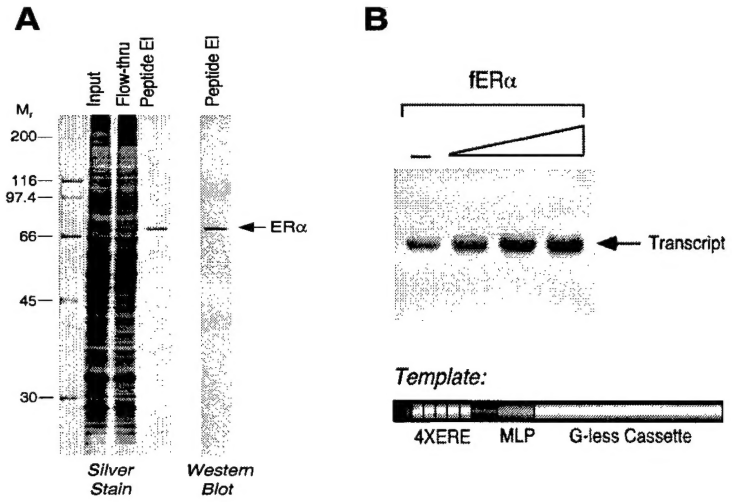

Figure 1. Purification of recombinant human ERα and analysis of its transcriptional activity. (A) recombinant ERα, bearing an amino terminal FLAG epitope tag, was purified directly from whole cell lysate of infected insect Sf21 cells by M2 (anti-FLAG) monoclonal antibody affinity chromatography. fERα protein thus purified was subjected to SDS-10%PAGE and analyzed by silver staining (left) to visualize protein purity or western blotting (right) with ERα-specific monoclonal antibody D12 to confirm protein identity. Input: infected insect whole cell lysate; Flow-thru: whole cell lysate following M2 antibody affinity chromatography; Peptide El: FLAG peptide eluate from M2 antibody affinity column. (B) In vitro transcription reactions were carried out using *Brca1-/-* MEF nuclear extract and the indicated template DNA bearing four copies of the consensus estrogen response element (ERE) upstream of the adenovirus major late core promoter (MLP) driving expression of a 200 bp G-less cassette. Where indicated, purified fERα was added at increasing concentrations (25, 50, and 100 ng in lanes 2-4). Transcription reactions were carried out in the presence of ATP, UTP, and 32P-CTP for 60 minutes at 30 degrees C. RNA transcripts were recovered by ethanol precipitation following deproteination, and recovered transcripts were resolved by denaturing polyacrylamide gel electrophoresis. Labeled transcripts were visualized by phosphorimager analysis.

consensus estrogen response element upstream of the adenovirus major late core promoter driving expression of a G-less cassette. We observed that fERα stimulated transcription, albeit weakly, from this reporter template in a dose-dependent manner, conclusively demonstrating the reconstitution of estrogen-independent ERα-directed transcriptional activation *in vitro*. However, a major caveat of these initial findings concerns the low level of activated transcription achieved in this system. At most, we have been able to achieve no more than several-fold stimulation of basal transcription by fERα. Future biochemical experiments will undoubtedly require more robust activation in this system, since we hope next to test the ability of recombinant BRCA1 protein to repress ligand-independent ERα-directed transcriptional activation.

In an attempt to achieve activation in this system that is at once considerably more robust and also subject to BRCA1-mediated repression, we have utilized an alternative approach based on the use of a hybrid VP16-GAL4-ERα transactivator. This chimeric protein includes the DNA-binding domain of the yeast transactivator GAL4 fused to the potent transcriptional activation domain of the Herpes simplex virus VP16 protein (Fig. 2B). VP16-GAL4 is by itself a potent constitutive activator in mammalian-based transcription systems from reporter templates beraring multimerized GAL4 DNA-binding sites upstream of a minimal core promoter. Previously, we and others have shown that translational fusion of VP16-GAL4 to the ERα hormone-binding domain (amino acids 251-595; Fig. 2C), effectively converts the VP16-GAL4 from a constitutive to an estrogen-dependent transactivator (VP16-GAL4-ERα) (3 and references therein). Furthermore, deletion analysis of this receptor chimera revealed that constitutive VP16-GAL4-ERα activity could be recovered by the removal of sequences within the ligand-binding domain of the ERα moiety, thereby implicating the ERα ligand-binding domain in ligand-independent transcriptional repression of a neighboring constitutive activation domain. To determine if this ligand-independent repression was, in fact, mediated mediated by BRCA1, we previously transfected the VP16-GAL4-ERα chimera along with a reporter template bearing GAL4 DNA binding sites into both BRCA1-proficient and BRCA1-deficient cells (3; Fig. 3). In BRCA1-proficient cells, the VP16-GAL4-ERα chimera exhibited minimal constitutive transactivation activity in the absence of estrogen (17 β-estradiol;

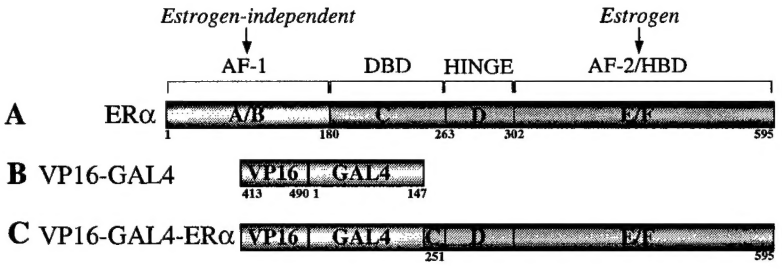

Figure 2. **(A)** Schematic representation of the functional domains identified in ERα, emphasizing the estrogen-independent and estrogen-dependent activation domains, AF-1 and AF-2, respectively. Numbers refer to amino acids that define the boundary of each domain. **(B)** Schematic representation of the constitutive activator VP16-GAL4 carrying amino acids 413-490 of the *Herpes simplex* virus virion protein 16 (corresponding to the VP16 activation domain) and amino acids 1-147 of the *S. cerevisiae* GAL4 protein (corresponding to the GAL4 DNA-binding domain). **(C)** Schematic representation of the ligand-dependent chimeric activator VP16-GAL4-ERα carrying amino acids 251-595 (corresponding to the hinge and hormone-binding domains of ERα) fused to VP16-GAL4.

E2); in response to E2, this level was dramtically increased to that approaching the potent VP16-GAL4 activator alone (3; Fig. 3A and B). By contrast, in BRCA1-deficient cells the VP16-GAL4-ERα chimera exhibited constitutive transactivation activity comparable to the VP16-GAL4 activator alone (Fig. 3C). The addition of E2 had a minimal effect on the elevated constitutive transactivation activity of the ERα chimera in BRCA1-deficient cells (data not shown) suggesting that the principle effect of E2 is to override a ligand-independent barrier to the transactivation activity of the chimeric receptor. This barrier is present in BRCA1-proficient, but not in BRCA1-deficient, cells. Importantly, we and others have previously mapped the BRCA1-binding domain on ERα to the ERα hormone-binding domain. Collectively, these results reveal the ERα ligand-binding domain to be a platform for the recruitment of BRCA1 from which the latter may confer ligand-independent repression on a linked activation domain. On the basis of these findings, we proposed a model in which BRCA1, along with an associated co-repressor(s) minimally including an HDAC activity, is recruited by unliganded, promoter-bound ERα to effectively silence the N-terminal constitutive AF-1 activation domain within ERα and thereby repress estrogen-responsive target gene transcription. Following estrogen stimulation, a ligand-induced conformational change within ERα could lead to enhanced affinity of the ERα for its cognate binding site and release of a BRCA1-containing repression complex, thereby liberating AF-1 and AF-2 to synergistically recruit coactivators and the RNA polymerase II holoenzyme to promote transcription (3).

Because the chimeric VP16-GAL4-ERα transactivator carries a potent transactivation domain whose constitutive activity is repressed by recruitment of BRCA1 through the ERα hormone-binding domain, we propose the use of this hybrid transactivator as an alternative approach to reconstitute BRCA1-mediated ligand-independent repression of ERα activity. To this end, we have generated recombinant six histidine-tagged-VP16-GAL4-ERα in *E. coli* (Fig. 4). Following its purificaton by metal affinity chromatography, we will examine the ability of this chimeric transactivator to stimulate transcription from a reporter template bearing multimerized GAL4 DNA-binding sites upstream of the minimal adenovirus major late core promoter in both *Brca1+/+ and Brca1-/-* MEF nuclear extracts. Based on the activity of this chimeric

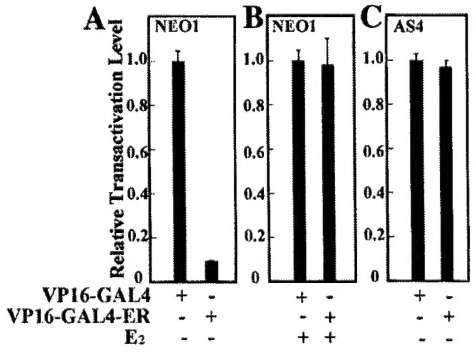

Figure 3. VP16-GAL4-ERα exhibits hormone-dependent activity in BRCA1-proficient (NEO1) cells and constitutive activity in BRCA1-deficient (AS4) cells. NEO1 (**A** and **B**) and AS4 (**C**) cells in estrogen-free media were transfected with a GAL4-E1B-Luc reporter plasmid along with (+) plasmids expressing either VP16-GAL4 or VP16-GAL4-ERα. Subseqently, transfected cells were either untreated (-) or treated (+) with 17-β-estradiol (E2; 10^{-7} M) prior to assay for luciferase activity.

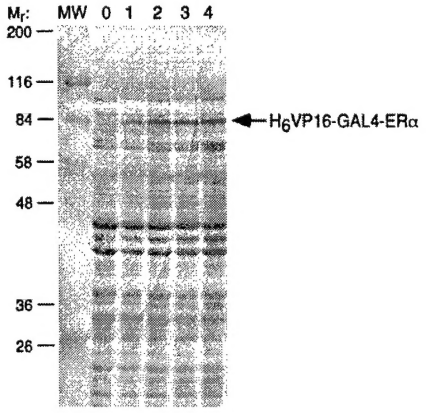

Figure 4. Expression analysis of recombinant six histidine-tagged (H_6) VP16-GAL4-ERα. Expression of H_6VP16-GAL4-ERα in BL-21 cells from the plasmid pQE32 was induced by the addition to cultures of 0.4 mM IPTG. Equivalent aliquots of cells were removed before induction (0) and at 1, 2, 3, and 4 hours after induction as indicated. Cells were lysed in SDS sample buffer, and cell lysates subsequently resolved by SDS-10% PAGE prior to analysis by coomassie blue staining to visualize expression of the 84 kDa H_6VP16-GAL4-ERα (indicated by the arrow).

transactivator in cultured *Brca1+/+ and Brca1-/-* MEFs, we expect that recombinant VP16-GAL4-ERα will exhibit significant constitutive activity in *Brca1-/-*, but not *Brca1+/+*, MEF nuclear extracts. Should this be the case, then we will examine the ability of recombinant BRCA1 protein to suppress the constitutive activity of this chimeric transactivator in *Brca1-/-* MEF nuclear extracts.

Technical Objective 2. To examine the role of estrogen-induced site-specific BRCA1 phosphorylation in the regulation of BRCA1-mediated ligand-independent ERα repression.

Task 1: ***Months 3-15:*** To identify estrogen-induced site-specifically phosphorylated residues on BRCA1. This will be done by immunopurification of BRCA1 from hormone-depleted MCF-7 human breast cancer cells stimulated with estrogen followed by both mass spectrometric analysis and immunoblot analysis using phosphopeptide-specific BRCA1 antibodies.

Recently, using our own previously published procedures (3), we have established conditions to reproducibly immunoprecipitate BRCA1 from both hormone-deprived and hormone-treated MCF-7 human breast cancer cells. In a typical small-scale experiment, MCF-7 cells are cultured in estrogen-free medium for a minimum of five days followed by treatment with or without 17-β-estradiolfor one hour. Subsequently, cell are harvested, lysed, and whole cell lysates then processed for immunoprecipitation using a combination of commercially available BRCA1-specific monoclonal antibodies (Ab-1, Ab-3, and Ab-4; Santa Cruz Biotechnologies). Recovered immunoprecipitates are then resolved by SDS-10% PAGE and processed by immunoblot analysis. Our results indicate specific immunoprecipitation of BRCA1. We are presently scaling up production of cultured cells in order to obtain sufficient quantities of immunoprecipitated BRCA1 protein for analysis by tandem mass spectrometry.

Technical Objective 3. To determine the role of BRCA1 in the control of paracrine growth signaling in the breast.

Task 1: ***Months 6-24:*** To compare the respective abilities of Brca1-intact and Brca1-mutated murine mammary epithelial cells to stimulate the proliferation of ERα-negative mammary epithelial cells through paracrine signaling in an estrogen-independent manner. This will be done by comparing the ability of conditioned serum-free medium obtained from *Brca1+/-* and *Brca1-/-* murine mammary

epithelial cells cultured in the absence or in the presence of estrogen for their potential to promote the growth of ER-negative MCF10A mammary epithelial cells in culture.

Experiments to establish primary murine mammary epithelial cells from both *Brca1+/-* and *Brca1-/-* mice are planned to begin within the next month.

KEY RESEARCH ACCOMPLISHMENTS

- Expression in and purification of FLAG epitope-tagged human estrogen receptor α (fERα) from insect Sf21 cells.
- Biochemical reconstitution of estrogen-independent ERα-directed transcriptional activation using nuclear extracts derived from *Brca1-/-* mouse embryo fibroblasts (MEFs) and highly purified recombinant fERα on an ERα-responsive reporter template *in vitro*.
- Construction and expression in *E. coli* of a recombinant VP16-GAL4-ERα hybrid transactivator protein for analysis of constitutive activity in *Brca1-/-* MEF nuclear extract.

REPORTABLE OUTCOMES

Reviews:

1. Trauernicht, A.M. and Boyer, T.G. BRCA1 and Estrogen Signaling in Breast Cancer. Breast Disease, Submitted. *Please refer to Appendix 2.*

Meeting Abstracts:

1. Trauernicht, A.M. and Boyer, T.G. (2004). Modulation of human estrogen receptor alpha (ERα) function by BRCA1. Nuclear Hormone Receptors. Keystone Symposia, Keystone, Colorado.

CONCLUSIONS

We have succeeded in the biochemical purification of recombinant human estrogen receptor α (ERα), and have further shown that recombinant ERα exhibits ligand-independent transcriptional activity in *Brca1*-deficient mouse embryo fibroblast (MEF) nuclear extract *in vitro*, thereby fulfilling Task 1 of Technical Objective 1. In an initial effort to achieve constitutive activation in this system that is at once considerably more robust than that observed with recombinant ERα and also subject to BRCA1-mediated repression, we have constructed and expressed a ligand-activated VP16-GAL4-ERα hybrid transactivator. Over the next two years, we will exploit this *in vitro* transcription system to study the mechanistic basis of BRCA1-mediated ligand-independent repression of ERα. Collectively, these studies should illuminate the molecular basis for the modulation of estrogen receptor function by BRCA1.

We have also establishedexperimentalconditions for thereproducible and specific immunopurification of BRCA1 from both estrogen-deprived and estrogen-stimulated MCF-7 human breast cancer cells, thus fulfilling a significant portion of Task 1 of Technical Objective 2. Over the next year, we will exploit these conditions to isolate sufficient quantities of endogenous BRCA1 protein for analysis by tandem mass spectrometry of estrogen-induced site-specifically phosphorylated residues. Collectively, we hope that these studies will offer possible insight into the tissue-specific tumor suppressor function of BRCA1 and suggest defined molecular targets for future intevention in breast cancer.

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3. Zheng, L., Annab, L.A., Afshari, C.A., Lee, W.-H., and Boyer, T.G. (2001). BRCA1 mediates ligand-independent transcriptional repression of the estrogen receptor. Proc. Natl. Acad. Sci. USA **98**: 9587-9592.

BRCA1 mediates ligand-independent transcriptional repression of the estrogen receptor

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Mutational inactivation of BRCA1 confers a cumulative lifetime risk of breast and ovarian cancers. However, the underlying basis for the tissue-restricted tumor-suppressive properties of BRCA1 remains poorly defined. Here we show that BRCA1 mediates ligand-independent transcriptional repression of the estrogen receptor α (ERα), a principal determinant of the growth, differentiation, and normal functional status of breasts and ovaries. In Brca1-null mouse embryo fibroblasts and BRCA1-deficient human ovarian cancer cells, ERα exhibited ligand-independent transcriptional activity that was not observed in Brca1-proficient cells. Ectopic expression in Brca1-deficient cells of wild-type BRCA1, but not clinically validated BRCA1 missense mutants, restored ligand-independent repression of ERα in a manner dependent upon apparent histone deacetylase activity. In estrogen-dependent human breast cancer cells, chromatin immunoprecipitation analysis revealed the association of BRCA1 with ERα at endogenous estrogen-response elements before, but not after estrogen stimulation. Collectively, these results reveal BRCA1 to be a ligand-reversible barrier to transcriptional activation by unliganded promoter-bound ERα and suggest a possible mechanism by which functional inactivation of BRCA1 could promote tumorigenesis through inappropriate hormonal regulation of mammary and ovarian epithelial cell proliferation.

Germline inactivation of the gene that encodes BRCA1 represents a predisposing genetic factor in ≈15–45% of hereditary breast cancers, and minimally 80% of combined hereditary breast and ovarian cancer cases (1). Functionally, BRCA1 has been implicated in the maintenance of global genome stability (2–4), and the underlying basis for this activity likely derives from its central role in the cellular response to DNA damage, wherein it controls both DNA damage repair and the transcription of DNA damage-inducible genes (5–14).

Because the DNA damage-induced signaling pathways that converge on BRCA1 are likely to be conserved in most cell types, BRCA1 is likely to occupy a fundamental and universally conserved role in the mammalian DNA damage response. Nonetheless, germ-line inactivation of BRCA1 leads predominantly to cancer of the breast and ovary, and the underlying basis for its tissue-restricted tumor-suppressive properties thus remains undefined.

At least two hypotheses have been proposed to explain the tissue-specific nature of BRCA1-mediated tumor suppression, both of which invoke a role for estrogen in either the initiation or promotion of tumor formation (15). According to one model, the tissue-specific tumor-suppressive properties of BRCA1 derive, at least in part, from its response to tissue-specific DNA damage. In this regard, certain oxidative metabolites of estrogen itself have been documented to be genotoxic in nature (16), and BRCA1 may therefore play a role in protecting breast and ovarian tissue from estrogen-induced DNA damage.

A second model, not mutually exclusive with the one described above, to account for the this tissue-specific tumor-suppressive function invokes a role for BRCA1 in the modulation of estrogen signaling pathways and, hence, the expression of hormone-responsive genes. In this regard, BRCA1 has been reported to inhibit estrogen-dependent transactivation by the estrogen receptor α (ERα) through its direct interaction with ERα (17, 18). BRCA1 has also been reported to enhance androgen-dependent transactivation by the androgen receptor, allelic variants of which modify cancer penetrance in BRCA1 mutation carriers (19–21). Based on its postulated role in the control of nuclear hormone signaling pathways, BRCA1 could therefore influence epithelial cell proliferation and, by implication, cancer risk in tissues such as breast and ovary.

Herein, we describe a role for BRCA1 in mediating ligand-independent transcriptional repression of the ERα. Initial efforts to elucidate the mechanistic basis for this repression reveal that BRCA1 represents a ligand-reversible barrier to transcriptional activation by unliganded promoter-bound ERα. These findings suggest a potential role for BRCA1 in the proliferative control of normal estrogen-regulated tissues and a potential basis by which its mutational inactivation could promote tumorigenesis through inappropriate hormonal responses.

BIOCHEMISTRY

Materials and Methods

Cell Culture. *p53−/−* (Brca1+/+) and *p53−/−; Brca1−/−* (Brca1−/−) mouse embryonic fibroblasts (MEFs) were cultured as described (14). Human MCF7 cells were maintained in DMEM supplemented with 10% FCS. Human BG-1-derived NEO1 and AS4 cell lines were maintained as described (22). Depletion of hormone ligands for nuclear/steroid receptor activation studies was achieved by cell culture in medium containing either 10% charcoal/dextran-treated serum (HyClone) or defined serum replacement 2 (Sigma).

Plasmids and Transfections. Transfection assays were performed by using the following conditions.

Reporter plasmids. Used at 0.5 μg each, including pTRE(F2)-TK-Luc, pGRE-TK-CAT, pERE-TK-Luc, or pPRE-TK-CAT (23); 0.5 μg of pGAL4-SV40-Luc containing five GAL4 DNA-binding sites upstream of the minimal simian virus 40 (SV40) promoter, driving expression of the luciferase reporter gene in the pGL2 vector (Promega); and 0.5 μg of pGAL4-E1B-Luc (24).

Receptor expression plasmids. Used at 1.0 μg each, including RSV-hTRβ, RSV-hGR, RSV-hERα, and RSV-hPRβ (23).

BRCA1 expression plasmids. Used at 1.0 μg each, including pcDNA3.1-BRCA1, pcDNA3.1-BRCA1-A1708E, pcDNA3.1-BRCA1-Q356R, and pcDNA3.1-BRCA1-A1708E/Q356R expressing either human wild-type BRCA1 or familial breast cancer-derived BRCA1 mutants (14).

Abbreviations: ERα, estrogen receptor α; MEF, mouse embryonic fibroblast; E2, 17β-estradiol; RT-PCR, reverse transcription–PCR; HDAC, histone deacetylase; ChIP, chromatin immunoprecipitation; AF-1, N-terminal ligand-independent activation function; AF-2, C-terminal ligand-inducible activation function.

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Chimeric activators. Used at 1.0 μg of GAL4-ERα, generated by an amino-terminal fusion of ERα with the GAL4 DNA-binding domain in pM3 (25); 0.1 μg of pVP16-GAL4 or pVP16-GAL4-ERα containing ERα amino acids 251–595, as described (26).

MEFs (6×10^4) or BG-1 cells (2×10^5) cultured in ligand-free medium were transfected by Lipofectin-based methods under serum-free conditions. Culture medium was replaced with fresh ligand-free medium 24 h after transfection, and 10^{-7} M 17β-estradiol (E2) or 330 nM trichostatin A was added as indicated. Cells were harvested 48 h after transfection for luciferase assay as described (14) or chloramphenicol acetyltransferase (CAT) assay by liquid scintillation counting (Promega).

Reverse Transcription (RT)-PCR Analysis. BG-1-derived cells were cultured in ligand-free medium for at least 5 days, and treated with 10^{-7} M E2 for 1 h as indicated. Approximately 15 μg of total cellular RNA was subjected to semiquantitative RT-PCR analysis following a procedure previously described for estrogen-responsive genes (27, 28).

Chromatin Immunoprecipitation (ChIP). MCF7 cells were cultured in ligand-free medium for at least 5 days and treated with 10^{-7} E2 for 1 h as indicated. ChIP assays were performed as described (29).

Antibodies. Antibodies used for soluble and chromatin immunoprecipitations and immunoblot analyses were as follows: BRCA1 (mAb 6B4); ERα (rabbit polyclonal antibody HC-20 or mouse mAb D-12, Santa Cruz Biotechnology); CtIP (mAb 19E8); TFIIH p89 (rabbit polyclonal antibody S-19, Santa Cruz Biotechnology); glutathione *S*-transferase (MAb 8G11); RNA polymerase II large subunit (mAb 8WG16); cathepsin D (rabbit polyclonal antibody 06-467, Upstate Biotechnology, Lake Placid, NY); pS2 (mouse mAb V3030, Biomeda, Hayward, CA); human progesterone receptor β (mouse mAb PriB-30, Santa Cruz Biotechnology); p84 (mAb 5E10).

Results

BRCA1 has been shown to modulate the ligand-dependent transcriptional activity of specific members of the nuclear hormone receptor family (17–20). However, endogenous BRCA1 present in the transfected cell lines used in previous studies precluded analysis of the effect of BRCA1 on the ligand-independent function of these receptors. Therefore, to more directly assess the role of BRCA1 in nuclear receptor transactivation without competition from endogenous BRCA1, we analyzed a panel of nuclear receptors for their respective ligand-independent transcriptional activities in Brca1-nullizygous MEFs.

A set of minimal thymidine kinase (TK) promoters, each under control of distinct hormone-response elements specific for either the human thyroid receptor β (TRβ), the glucocorticoid receptor (GR), the ERα, or the progesterone receptor β (PRβ) were individually tested for their respective abilities to direct expression of a reporter gene in the absence or presence of each corresponding receptor (absent ligand) after transfection into Brca1-proficient (Brca1+/+) or Brca1-deficient (Brca1−/−) MEFs (14). Unexpectedly, we observed significant ligand-independent activation of reporter gene expression directed by both the progesterone receptor β and the ERα in Brca1-deficient MEFs compared with Brca1-proficient MEFs (Fig. 1*A*). By contrast, no ligand-independent stimulation of reporter activity directed by either the thyroid receptor β or the glucocorticoid receptor could be observed in Brca1-deficient MEFs (Fig. 1*A*). Interestingly, although E2 activated the ERα in both Brca1-proficient and Brca1-deficient MEFs, the relative level of induction observed in Brca1-deficient MEFs was diminished 2-fold

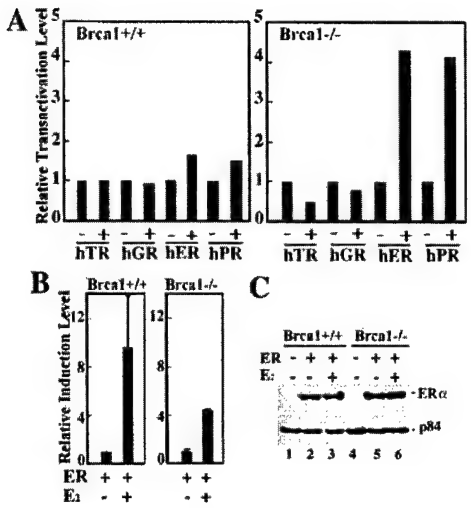

Fig. 1. BRCA1 mediates ligand-independent repression of the receptors for estrogen and progesterone. (*A*) Brca1+/+ and Brca1−/− MEFs in hormone-free media were transfected with reporter plasmids (pTK-Luc or pTK-CAT) carrying response elements specific for individual hormone receptors without (−) or with (+) plasmids expressing the human thyroid receptor β (hTR), glucocorticoid receptor (hGR), estrogen receptor α (hER), or progesterone receptor β (hPR). Transfections performed without (−) receptor expression plasmids were performed instead with a molar equivalent of the backbone expression plasmid pRSV. The relative transactivation level represents the fold-increase in transfected reporter gene activity measured in cells cotransfected with a specific receptor expression plasmid relative to the level of transfected reporter gene activity measured in cells cotransfected with the backbone pRSV expression plasmid. Reporter gene activity was first normalized to β-galactosidase activity obtained by cotransfection of an internal control pSV40-β-gal expression plasmid as described (14). Expression of the pSV40-β-gal plasmid was not affected by the absence of presence of BRCA1 or any of the nuclear hormone receptors analyzed (data not shown). (*B*) Brca1+/+ and Brca1−/− MEFs in estrogen-free media were transfected with pERE-TK-Luc carrying three copies of the consensus estrogen response element (ERE) with (+) pRSV-ERα in the absence (−) or presence (+) of E2 (10^{-7} M) before assay for luciferase activity. The relative induction level represents the relative transactivation level measured in the presence of E2 divided by the relative transactivation level measured in the absence of E2. (*C*) Brca1+/+ (lanes 1–3) and Brca1−/− (lanes 4–6) MEFs either untransfected (lanes 1 and 4) or transfected (lanes 2, 3, 5, and 6) with an ERα-expressing vector were lysed, and immunoprecipitated ERα was immunoblotted with ERα-specific antibodies (*Upper*). Immunoblot analysis of the nuclear matrix protein p84 (*Lower*) indicates that nearly equivalent amounts of each cell lysate were used in the immunoprecipitations.

relative to Brca1-proficient MEFs (Fig. 1*B*). We confirmed by immunoblot analysis that the transfected ERα was expressed equivalently in BRCA1-proficient and BRCA1-deficient MEFs, thus excluding the possibility that differences in receptor activity derive from differences in receptor protein expression (Fig. 1*C*).

Ectopic expression of wild-type BRCA1 in Brca1-deficient MEFs repressed ligand-independent activation directed by ERα (Fig. 2*A*). Likewise, a BRCA1 derivative carrying a familial breast cancer-derived missense mutation in the ring finger (C64G) also repressed ligand-independent activation by ERα (Fig. 2*A*). By contrast, BRCA1 derivatives carrying familial breast cancer-derived missense mutations in either an exon 11-encoded region that binds Rad50 and the transcriptional repressor ZBRK1 (Q356R) or the C-terminal BRCT domain (A1708E) abolished the ability of BRCA1 to repress ligand-independent transactivation directed by ERα (Fig. 2*A*). Differences in the transcriptional repression activities of the various BRCA1 mutant derivatives could not be attributed to differences in their respective levels of expression because each of the BRCA1 mutant derivatives was expressed at a level comparable

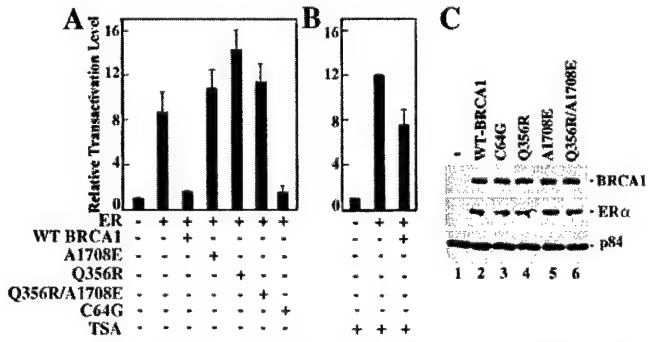

Fig. 2. Ectopic expression of wild-type BRCA1 in Brca1-deficient MEFs restores ligand-independent repression of ERα transactivation in a histone deacetylase (HDAC)-dependent manner. (*A* and *B*) Brca1−/− MEFs in estrogen-free media were transfected with pERE-TK-Luc without (−) or with (+) pRSV-ERα, pCDNA3.1-BRCA1 expressing wild-type human BRCA1 (WT), or pCDNA3.1-BRCA1 derivatives bearing missense mutants A1708E, Q356R, A1708E/Q356R, or C64G before assay for luciferase activity. Where indicated, trichostatin A (TSA; 330 nM) was also included. (*C*) Brca1−/− MEFs in estrogen-free media were untransfected (lane 1) or cotransfected with expression vectors for ERα and either wild-type BRCA1 (lane 2) or various BRCA1 mutant derivatives (lanes 3–6) as indicated. Cells were lysed, and immunoprecipitated BRCA1 and ERα were subjected to immunoblot analysis using antibodies specific for BRCA1 (*Top*) or ERα (*Middle*). Immunoblot analysis of the nuclear matrix protein p84 (*Bottom*) indicates that nearly equivalent amounts of each cell lysate were used in the immunoprecipitations.

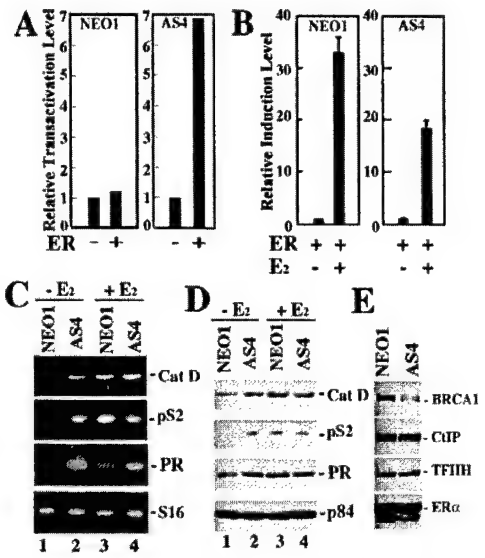

Fig. 3. Reduced BRCA1 expression in BG-1 human ovarian adenocarcinoma cells is accompanied by increases in estrogen-independent expression of estrogen-responsive genes. (*A*) Retroviral vector-infected (NEO1) and BRCA1 antisense-infected (AS4) BG-1 cell clones in estrogen-free media were transfected with pERE-TK-Luc without (−) or with (+) pRSV-ERα before assay for luciferase activity. (*B*) NEO1 and AS4 cells in estrogen-free media were transfected with pERE-TK-Luc with (+) pRSV-ERα in the absence (−) or presence (+) of E2 (10^{-7} M) before assay for luciferase activity. (*C*) NEO1 (lanes 1 and 3) or AS4 (lanes 2 and 4) cells in estrogen-free media were either untreated (lanes 1 and 2) or treated (lanes 3 and 4) with E2 (10^{-7} M) for 1 h. Cells were harvested and processed for semiquantitative RT-PCR analysis using primers specific for the estrogen-responsive cathepsin D (Cat D), pS2, and progesterone receptor genes, as well as the estrogen-nonresponsive ribosomal S16 gene. (*D*) NEO1 (lanes 1 and 3) or AS4 (lanes 2 and 4) cells (5×10^6) in estrogen-free media were either untreated (lanes 1 and 2) or treated (lanes 3 and 4) with E2 (10^{-7} M) for 24 h. Culture medium was concentrated 10-fold by using a Centriprep YM-3 device, and 1/10th of the concentrate was resolved by SDS/15%PAGE and processed for immunoblot analysis using antibodies specific for pS2. Cells were also lysed in RIPA buffer, and 1/10th of the lysate was subjected to immunoblot analysis using antibodies specific for progesterone receptor β (PR), cathepsin D (Cat D), or nuclear matrix protein p84, which served as an internal loading control. (*E*) Whole cell lysates derived from NEO1 and AS4 cells were resolved by SDS/10%PAGE and processed for immunoblot analysis using antibodies specific for BRCA1, CtIP, and the p89 subunit of the transcription factor IIH (TFIIH), the latter two of which served as internal loading controls. The ERα-positive status of these cells was verified by using an ERα-specific rabbit polyclonal antibody. Densitometric quantitation of the immunoblot and normalization to the CtIP and TFIIH signals revealed BRCA1 expression to be reduced by 70% in AS4 cells compared with NEO1 cells.

to wild-type BRCA1 (Fig. 2*C*). BRCA1-mediated, ligand-independent repression of ERα was largely reversed by trichostatin A, implicating histone deacetylase (HDAC) activity in this process (Fig. 2*B*). Collectively, these results reveal a function for BRCA1 as a repressor of ligand-independent, ERα-mediated transactivation.

To confirm these results in a biologically relevant cell type, we analyzed the ligand-independent activity of ERα in human ovarian adenocarcinoma BG-1 cells, which are ERα-positive and estrogen-dependent for growth (30). Previously, Annab *et al.* (22) described the generation of independent BG-1 clonal cell lines that support stably reduced BRCA1 mRNA and protein levels by retroviral-mediated BRCA1 antisense delivery. We tested the ability of ERα to direct ligand-independent transcription of the ERE-TK-Luc reporter gene after transfection into either a control retroviral vector-infected BG-1 clonal cell line (NEO1) or, alternatively, a BRCA1 antisense-infected BG-1 clonal cell line (AS4) exhibiting severely reduced BRCA1 expression levels (Fig. 3*E*; ref. 22). Consistent with the results obtained in MEF cells, ERα exhibited significantly increased ligand-independent activity in BRCA1-deficient AS4 cells compared with BRCA1-proficient NEO1 cells (Fig. 3*A*). We also observed a 2-fold reduction in the relative level of E2-mediated induction of reporter gene activity in AS4 cells compared with NEO1 cells, once again consistent with the results obtained in MEF cells (Fig. 3*B*). These results confirm that in a biologically relevant epithelial cell type, BRCA1 can mediate repression of ligand-independent ERα transactivation activity.

To determine whether the reduced BRCA1 expression levels in AS4 cells could be correlated with an increase in the ligand-independent expression of endogenous estrogen-responsive genes, we performed a direct comparative analysis of NEO1 and AS4 cells with respect to their ligand-independent expression of several estrogen-responsive genes. Individual monolayer cultures of NEO1 and AS4 cells were grown in the absence of estrogen for 5 days followed by the addition of either no hormone or, alternatively, E2 (10^{-7} M) for 1 h. Subsequently, cells were harvested and analyzed by semiquantitative RT-PCR for the expression levels of the endogenous estrogen-responsive pS2, cathepsin D, and progesterone receptor genes.

Relative to the expression level of an internal control ribosomal S16 gene, we observed increases in the ligand-independent expression levels of the pS2, cathepsin D, and progesterone receptor genes of 3-, 5-, and 9-fold, respectively, in BRCA1-deficient AS4 cells compared with BRCA1-proficient NEO1 cells (Fig. 3*C*). Interestingly, although the addition of E2 stimulated transcription of the pS2, cathepsin D, and the progesterone receptor genes in NEO1 cells, no such E2-dependent increase in the transcription of these genes could be observed in AS4 cells (Fig. 3*C*). Qualitatively similar results were observed at the protein level by immunoblot analysis. Relative to the level of an internal control protein (nuclear matrix protein p84), E2-independent increases in the steady-state levels of the pS2, cathepsin D, and progesterone receptor proteins could be observed in AS4 cells compared with NEO1 cells (Fig. 3*D*). Furthermore, although the addition of E2 elevated the steady-

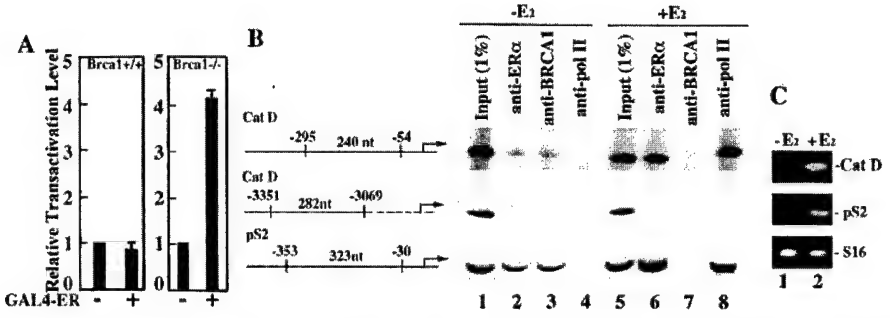

Fig. 4. BRCA1 represses unliganded promoter-bound ERα-mediated transactivation. (*A*) Brca1+/+ and Brca1−/− MEFs were transfected with a pGAL4-SV40-Luc reporter plasmid either without (−) or with (+) a pGAL4-ERα expression plasmid before assay for luciferase activity. (*B*) Schematic diagram of the cathepsin D (Cat D) and pS2 gene regions targeted for ChIP analysis. Negative numbers refer to sequence coordinates that delimit PCR amplicons defined by gene-specific primer pairs relative to the transcription initiation site (right-angled arrow). Numbered nucleotides (nt) refer to the expected sizes of PCR-amplified products. MCF-7 cells, cultured the absence of estrogen, were treated without (−E2) or with (+E2) E2 (10^{-7} M) for 1 h. Soluble chromatin was prepared and subjected to immunoprecipitation by using monoclonal antibodies specific for ERα (anti-ERα), BRCA1 (anti-BRCA1), or the RNA polymerase II large subunit (anti-pol II). Immunoprecipitated DNA was PCR-amplified by using primers that span the indicated regions of the cathepsin D and pS2 gene promoters. Input (1%) of the soluble chromatin subjected to immunoprecipitation was PCR-amplified directly by using each primer pair as indicated. (*C*) MCF-7 cells, cultured in the absence of estrogen, were treated without (−E2) or with (+E2) E2 (10^{-7} M) for 1 h before harvest and processing for semiquantitative RT-PCR analysis using primers specific for the estrogen-responsive cathepsin D (Cat D) and pS2 genes, as well as the estrogen-nonresponsive ribosomal S16 gene.

state level of each of these proteins in NEO1 cells, no such E2-dependent increase could be observed in AS4 cells (Fig. 3*D*). Quantitative differences between RT-PCR and immunoblot analyses could reflect the influence of posttranscriptional regulatory processes. Nonetheless, RT-PCR and immunoblot analyses both reveal that the ligand-independent expression of endogenous ERα-target genes is increased in BRCA1-deficient cells. Collectively, these results implicate BRCA1 in the ligand-independent repression of endogenous estrogen-responsive genes.

To explore the mechanism by which BRCA1 mediates ligand-independent repression of ERα, we first determined whether BRCA1 could interact with unliganded ERα *in vivo* by coimmunoprecipitation of the two proteins in human breast cancer MCF7 cells cultured in the absence of estrogen. Consistent with previous results (18), BRCA1 could be specifically coimmunoprecipitated with unliganded ERα, thus demonstrating that the two proteins can interact *in vivo* in a ligand-independent manner (data not shown).

To explore the possibility that BRCA1 represses the transactivation function of promoter-bound, unliganded ERα, we first tested the effect of BRCA1 on the ligand-independent transcriptional activity of ERα tethered to the yeast GAL4 DNA-binding domain by using a reporter template bearing GAL4 DNA-binding sites. This approach permitted us to assess the effect of BRCA1 on the transactivation function of unliganded ERα independent of any effects that BRCA1 might have on the DNA-binding activity of unliganded ERα. GAL4-ERα was cotransfected along with a GAL4-SV40-luciferase reporter template into Brca1-proficient and Brca1-deficient MEFs. We observed significant ligand-independent stimulation of reporter activity in Brca1-deficient, but not in Brca1-proficient, MEFs (Fig. 4*A*), suggesting one mechanism by which BRCA1 mediates ligand-independent repression of ERα is through direct repression of the DNA-bound receptor.

To confirm this observation under biologically relevant conditions *in vivo*, we used ChIP analyses to determine whether BRCA1 can be recruited directly to estrogen-responsive promoters in the absence of ligand. MCF-7 cells were grown in the absence of estrogen for 5 days followed by the addition of either no hormone or, alternatively, E2 (10^{-7} M) for 1 h. Promoter occupancy before and after E2 treatment at the estrogen response elements within the endogenous pS2 and cathepsin D gene promoters by ERα, BRCA1, and RNA polymerase II was then monitored by ChIP using antibodies specific for each of the three proteins and semiquantitative PCR with primers flanking the estrogen response elements of the pS2 and cathepsin D promoters. In the absence of E2, ERα could be detected in association with both the pS2 and cathepsin D promoters, and this level was increased dramatically by the addition of E2 (Fig. 4*B*, lanes 2 and 6). Strikingly, we also observed pS2 and cathepsin D promoter occupancy by BRCA1 in the absence of E2, and a reduction in such occupancy after E2 treatment (Fig. 4*B*, lanes 3 and 7). By contrast, RNA polymerase II could be detected only following, but not before, E2 treatment, consistent with its ligand-dependent recruitment concomitant with transcriptional activation of the pS2 and cathepsin D genes (Fig. 4*B*, lanes 4 and 8 and *C*, lanes 1 and 2). The specificity of factor association within the estrogen-responsive region of the pS2 and cathepsin D promoters was confirmed by ChIP analysis using antibodies specific for ZBRK1, a sequence-specific DNA-binding transcriptional repressor that does not bind to pS2 or cathepsin D promoter sequences (14). ZBRK1-specific antibodies failed to immunoprecipitate pS2 and cathepsin D promoter sequences (data not shown). Further specificity of the ChIP assay was demonstrated by the inability to detect occupancy by ERα, BRCA1, or RNA polymerase II of a region ≈3 kb upstream of the cathepsin D promoter (Fig. 4*B*). These results thus reveal the association of BRCA1 with unliganded ERα at endogenous estrogen-responsive promoters under physiologically relevant conditions *in vivo*.

Like other steroid receptors, ERα contains two transactivation domains, an N-terminal ligand-independent activation function (AF-1) that is targeted by a variety of steroid-independent cell-signaling pathways, and a C-terminal ligand-inducible activation function (AF-2) that resides within the receptor ligand-binding domain (31, 32). Previous analyses of ERα suggest a model whereby repressive factors binding to sequences within its C-terminal ligand-binding domain repress constitutively active AF-1 in the absence of an agonist or in the presence of an antagonist (26, 33). To determine whether ligand-independent repression of ERα by BRCA1 is mediated through the ERα ligand-binding domain, we tested the ligand-independent activity of a VP16-GAL4-ERα receptor chimera after its expression in both BRCA1-proficient and BRCA1-deficient BG-1 clonal cell lines. This chimera encodes ERα amino acids 251–595, including the hinge region and the ligand-binding domain, fused C-terminally to the hybrid transactivator VP16-GAL4 (26).

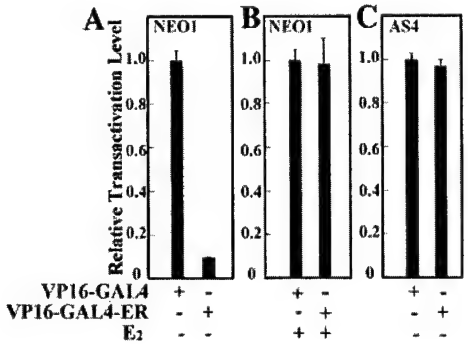

Fig. 5. VP16-GAL4-ERα exhibits hormone-dependent activity in BRCA1-proficient cells and constitutive activity in BRCA1-deficient cells. NEO1 (*A* and *B*) and AS4 (*C*) cells in estrogen-free media were transfected with a GAL4-E1B-Luc reporter plasmid along with (+) plasmids expressing either VP16-GAL4 or VP16-GAL4-ERα. Subsequently, transfected cells were either untreated (−) or treated (+) with E2 (10^{-7} M) before assay for luciferase activity.

Previously, deletion analysis of this receptor chimera revealed that constitutive VP16-GAL4-ERα activity could be recovered by the removal of sequences within the ligand-binding domain of the ERα moiety, thereby implicating the ERα ligand-binding domain in ligand-independent transcriptional repression of a neighboring constitutive activation domain (26). To determine whether this ligand-independent repression is mediated by BRCA1, we transfected the VP16-GAL4-ERα chimera along with a reporter template bearing GAL4 DNA binding sites into both BRCA1-proficient NEO1 cells and BRCA1-deficient AS4 cells. In NEO1 cells, the VP16-GAL4-ERα chimera exhibited minimal constitutive transactivation activity in the absence of E2; in response to E2, this level was dramatically increased to one approaching that of the potent VP16-GAL4 activator alone (Fig. 5 *A* and *B*). By contrast, in AS4 cells the VP16-GAL4-ERα chimera exhibited constitutive transactivation activity comparable to that exhibited by the VP16-GAL4 activator alone (Fig. 5*C*). The addition of E2 had a minimal effect on the elevated constitutive transactivation activity of the ERα chimera in AS4 cells (data not shown), suggesting that the principle effect of E2 is to override a ligand-independent barrier to the transactivation activity of the chimeric receptor. This barrier is present in NEO1 cells, but deficient in AS4 cells. Similar results were also observed by using isogenic Brca1-proficient and Brca1-deficient MEFs, eliminating the possibility that cell type-specific peculiarities contribute to the differential transactivation properties of the VP16-GAL4-ERα chimera in the presence and absence of BRCA1 (data not shown). Collectively, these results reveal the ERα ligand-binding domain to be a platform for the recruitment of BRCA1 from which the latter may confer ligand-independent repression on a linked activation domain. Hence, we conclude that BRCA1-mediated ligand-independent repression of ERα is likely to be mediated through the ERα ligand-binding domain.

Discussion

Recently, BRCA1 has been proposed to inhibit the ligand-dependent transcriptional activity of ERα through a direct interaction between the two proteins (18). Our current analysis of ERα transcriptional activity in Brca1-nullizygous MEFs revealed BRCA1 to be a ligand-reversible barrier to transcriptional activation by unliganded ERα. The biological relevance of this finding is further strengthened by the observation that BRCA1 also mediates ligand-independent repression of the ERα in human ovarian adenocarcinoma cells.

The underlying mechanism by which BRCA1 mediates ligand-independent repression of ERα transcriptional activity appears to involve targeted recruitment by unliganded, promoter-bound ERα of a BRCA1-associated HDAC activity. This conclusion is based first on the observation that the HDAC inhibitor trichostatin A can effectively reverse ligand-independent repression mediated by BRCA1 and, second, on the results of ChIP analyses, which revealed the association of unliganded ERα with BRCA1 on endogenous estrogen-response elements *in vivo*. A likely target of BRCA1-mediated ligand-independent ERα repression is the constitutive AF-1 activation domain within ERα. Previous studies have indicated that antagonist-bound AF-2 can repress AF-1 activity through the recruitment of the nuclear corepressor N-CoR (33), whereas the ligand-binding domain of unliganded ERα can repress a linked heterologous activation domain in a ligand-reversible manner, presumably by the recruitment of a soluble corepressor (26). Our observation that an estrogen-dependent VP16-GAL4 chimeric transactivator carrying the ERα ligand-binding domain exhibits constitutive activity in BRCA1-deficient, but not in BRCA1-proficient BG-1 cells, reveals the ERα ligand-binding domain to be a potential site of BRCA1 recruitment for ligand-independent repression of a linked activation domain. Hence, BRCA1 could be recruited to the ERα ligand-binding domain as part of a larger repression complex to silence AF-1 function in the absence of ligand. The recent report of a direct interaction between BRCA1 and the ERα ligand-binding domain (18) lends additional support to this model.

Should BRCA1 function to inhibit the ligand-dependent transcriptional activity of ERα (17, 18), it seems unlikely to do so through a mechanism that involves promoter-bound ERα. Our ChIP analysis revealed the association of BRCA1 with ERα at endogenous estrogen-response elements before, but not after, estrogen stimulation. Thus, we favor a model in which BRCA1, along with an associated corepressor(s) that minimally includes an HDAC activity, is recruited by unliganded, promoter-bound ERα to effectively silence the constitutive AF-1 activation domain and thereby repress estrogen-responsive target gene transcription. After estrogen stimulation, a ligand-induced conformational change within ERα could lead to enhanced affinity of the ERα for its cognate binding site and release of a BRCA1-containing repression complex, thereby liberating AF-1 and AF-2 to synergistically recruit coactivators and the RNA polymerase II holoenzyme to promote transcription (29). It is also possible that BRCA1 could function additionally as a barrier to the productive association of either unliganded and/or liganded ERα with promoter DNA, and this could underlie the previous observation that BRCA1 can inhibit ligand-dependent ERα transactivation (17, 18).

Interestingly, we observed that a deficiency of BRCA1 also leads to a reduction in the relative level of E2-mediated ERα activation. In both Brca1-nullizygous MEFs and BRCA1-deficient BG-1 (AS4) cells, the relative level of E2-mediated activation of a transfected ERα-responsive reporter gene was diminished when compared with Brca1-proficient cells. Furthermore, in AS4 cells, the endogenous estrogen-response genes that we monitored exhibited increased estrogen-independent expression and little or no estrogen-dependent stimulation when compared with BRCA1-proficient BG-1 (NEO1) cells. It is possible that the expression of these genes is largely derepressed in a BRCA1-deficient background and cannot therefore be increased substantially in response to estrogen.

Previously, Annab *et al.* (22) demonstrated that relative to parental or retroviral vector-infected BG-1 cell clones, BRCA1 antisense-infected BG-1 cell clones exhibit enhanced estrogen-independent growth in culture (22). Furthermore, BG-1 clone AS4, which exhibits severely reduced BRCA1 expression levels, exhibited increased tumorigenicity in ovariectomized nude mice compared with the retroviral vector-infected NEO1 cell clone (22). These observations suggest that forced reduction of

BRCA1 in BG-1 ovarian adenocarcinoma cells may influence estrogen-independent growth both *in vitro* and *in vivo*. Our observation that AS4 cells support significant increases in the estrogen-independent expression levels of different ERα-target genes compared with BRCA1-proficient NEO1 cells may provide a mechanistic basis for the estrogen-independent growth advantages that AS4 cells exhibit.

The finding that BRCA1 can function as a ligand-reversible barrier to transcriptional activation by unliganded ERα suggests the potential involvement of BRCA1 in the proliferative control of normal estrogen-regulated tissues. Thus, mutational inactivation of BRCA1 could result in persistent expression of estrogen-responsive genes in the absence of threshold levels of estrogenic stimulation. In this way, inappropriate hormonal responses brought about by BRCA1 mutation might possibly promote the proliferation of transformation-initiated cells.

Previous analyses have revealed that a significant proportion of BRCA1-associated breast tumors are negative for ERα expression (34). However, the loss of ERα expression in BRCA1-associated tumors is likely to represent a relatively late event in breast tumor progression, one that may have occurred after any proliferative advantages conferred upon transformation-initiated cells by homozygous BRCA1 mutation have ensued. Possibly, the down-regulation of ERα expression in BRCA1-mutated tumors could derive in part from negative feedback control enlisted by BRCA1-mutated breast epithelial cells to restrict the promiscuous expression of estrogen-responsive genes. Future studies should illuminate the mechanistic basis for BRCA1-mediated transcriptional repression of ERα and clarify its functional role in the larger network of hormone signaling pathways that control the growth, differentiation, and homeostasis of breast and ovary.

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Appendix 2

BRCA1 and estrogen signaling in breast cancer

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ABSTRACT

Since the gene encoding BRCA1 was first cloned in 1994, researchers have sought to establish the molecular basis for its linkage to breast and ovarian cancer. As universal functions for this protein have emerged, questions persist concerning how its disruption can elicit cancer in a tissue- and gender-specific manner. Here, we review evidence that a functional interrelationship between BRCA1 and estrogen signaling may be involved in breast tumorigenesis.

INTRODUCTION

The National Cancer Institute reports that 1 in 8 women will develop breast cancer during her lifetime (*1*). The high incidence of this disease therefore renders identification of risk factors and underlying causes a major focus of basic and clinically applied research. Although multiple factors influence a woman's lifetime risk for the development of breast cancer, family history is one of the most powerful prognostic indicators. Indeed, approximately 10% of all breast cancer cases can be linked to heritable transmission of an autosomal dominant allele (*2*).

Through linkage analysis of multiple families affected by early-onset breast and ovarian cancer, the first breast cancer susceptibility gene, *BRCA1*, was mapped to chromosome 17q21 in 1990 and cloned four years later (*3,4*). At the same time, *BRCA2* was mapped to chromosome 13q and cloned shortly thereafter (*5,6*). Mutations in *BRCA1* are believed to account for 60-80% of hereditary breast and ovarian cancer cases and up to 15-20% of hereditary breast cancers only (*7*). *BRCA2* mutations are linked to a similar percentage of inherited female breast cancers, but in contrast to mutations in *BRCA1*, they also predispose males to breast cancer (*6*). Together, defects in these two genes account for approximately 40% of inherited breast cancers (*7*).

Germline inactivation of a single copy of *BRCA1* or *BRCA2* is sufficient to predispose an affected individual to cancer, while cancer onset is invariably accompanied by loss of the remaining allele (*8*). Thus, *BRCA1* and *BRCA2* are tumor susceptibility genes that normally function to suppress tumor formation.

Because somatic mutations in *BRCA1* and *BRCA2* are rare, it was originally assumed that neither gene plays an important role in the development of sporadic breast and ovarian cancers. However, recent studies indicate that epigenetic disruption of *BRCA1* or *BRCA2* function could represent a significant etiologic factor in sporadic disease (*9*).

While genetic or epigenetic inactivation of *BRCA1* or *BRCA2* is thus sufficient to induce cancer formation, emerging evidence suggests that disruption of these genes may induce tumorigenesis through distinct molecular pathways. First, *BRCA1*- and *BRCA2*-associated breast tumors are distinguishable histopathologically; whereas *BRCA1*-associated tumors are often high-grade cancers characterized by a high mitotic index and lymphocytic infiltrate, *BRCA2*-associated tumors are heterogeneous, relatively high grade, and generally display substantially less tubule formation (*10,11*). Second, *BRCA1*-associated tumors are generally characterized by estrogen and progesterone receptor negativity, while *BRCA2*-associated tumors more commonly express these hormone receptors (*12*). Finally, distinct gene expression profiles characteristic of *BRCA1*- and *BRCA2*-associated tumors indicate distinct molecular phenotypes (*13-15*). Together, these findings imply that BRCA1 and BRCA2 may participate in distinct pathways leading to breast and ovarian carcinogenesis.

The scope of this review will be restricted principally to the role of BRCA1 in breast cancer. Following a brief summary of recent advances that extend our understanding of its generic biological function and regulation, we discuss recent evidence that links BRCA1 to

estrogen signaling and consider the possibility that this link represents an important etiologic factor in breast cancer development. The reader is referred to several recent reviews for a more comprehensive discussion of BRCA1 and BRCA2 in breast and ovarian cancer (*16-19*).

BRCA1 STRUCTURE AND EXPRESSION

The gene encoding BRCA1 spans more than 100 kb of genomic DNA and comprises 24 exons, 22 of which encode a full-length isoform of 1863 amino acids (*4*). In addition, several smaller BRCA1 isoforms of variable size and tissue-specific expression patterns are produced, primarily through alternative splicing of exons 1 and 11 (*20*). Exon 11, the largest exon, encodes roughly 60% of the protein and includes two putative nuclear localization sequences (*20*).

The full-length BRCA1 isoform is a 220 kDa nuclear phosphoprotein (*21,22*). At its amino terminus, BRCA1 harbors a structurally conserved zinc-binding RING finger domain (*4*). Consistent with the recent observation that otherwise diverse proteins harboring RING fingers can function as ubiquitin protein ligases, the BRCA1 RING finger itself has been shown to exhibit ubiquitin ligase activity that is greatly stimulated by heterodimerization with a partner RING finger protein BARD1 (*23,24*).

At its carboxyl terminus, BRCA1 carries two tandem copies of the **BRCT** (**BR**CA1 **C**arboxyl **T**erminus) domain (*25*). An autonomous folding unit defined by conserved clusters of hydrophobic amino acids, the BRCT domain is found in a diverse group of DNA repair and cell cycle control proteins, and likely functions as a protein interaction surface. Consistent with this notion, the BRCT domain in BRCA1 represents an interface for a variety of proteins that are critical for its function in transcription control (*26*).

Developmentally, *BRCA1* is expressed in all tissues, but most highly in rapidly proliferating and differentiating cellular compartments; in the mouse mammary gland, these compartments include the terminal end buds during puberty and differentiating alveoli during pregnancy (*27,28*). In addition, the unique temporal and tissue-specific pattern of BRCA1 expression during prenatal development of the human mammary gland is consistent with a role for BRCA1 in mammary gland morphogenesis and differentiation (*29*). Furthermore, BRCA1 expression is upregulated in mammary epithelial cells induced to differentiate in vitro (*30*), while forced reduction of BRCA1 expression attenuates the in vitro differentiation of mammary epithelial cells, but not muscle or neuronal cells (*31*). Taken together, these data imply a fundamental role for BRCA1 in the control of mammary epithelial cell differentiation, although its precise role in this process remains to be established.

The induction of BRCA1 expression coincident with differentiation in the mammary gland is not inconsistent with its possible regulation by estrogen, a major determinant of the growth and differentiation of mammary epithelial cells. In fact, data from early studies appeared to support this possibility. In the mammary gland, BRCA1 expression was shown to be upregulated following treatment of ovariectomized mice with estradiol and progesterone (*32*), while estrogen-responsive MCF-7 and BT20T human breast cancer cells, cultured in the absence of estrogen, exhibited reduced BRCA1 expression levels that could be reversed by the addition of estrogen (*33,34*). However, subsequent studies revealed that estrogen indirectly regulates BRCA1 expression by virtue of its mitogenic activity in promoting G1-S phase progression through the cell cycle. In cultured human mammary epithelial and cancer cell lines, BRCA1 has been shown to be expressed cyclically; *BRCA1* mRNA and protein expression levels peak during G1/S or early S-phase of the cell cycle concomitant with BRCA1 phosphorylation (*21,22*). The

delayed kinetics of BRCA1 induction following estrogen treatment parallels DNA synthesis and is therefore inconsistent with direct transcriptional regulation of BRCA1 by estrogen (*35*). Furthermore, BRCA1 is expressed independently of hormonal stimulation in the mouse ovary and similarly in estrogen receptor-proficient and -deficient mice (*36*). Collectively, these findings indicate that BRCA1 is not itself a direct transcriptional target of estrogen.

BRCA1 FUNCTION

Insight into the biological function of BRCA1 has come from analyses of cells derived from BRCA1-mutant human breast tumors and embryos of mice carrying targeted deletions of the *BRCA1* gene. Invariably, BRCA1-deficient cells develop gross chromosomal abnormalities, typified by breaks, aberrant mitotic exchanges and aneuploidy (*37,38*). These findings thus reveal BRCA1 to be cellular caretaker that suppresses genomic instability. Emerging evidence suggests that the underlying basis for this caretaker activity likely derives from the role of BRCA1 as a conduit in the cellular DNA damage response, wherein it serves to couple DNA damage-induced signals to downstream responses including DNA damage repair and cell cycle checkpoint activation.

BRCA1 and DNA Damage Repair

Evidence to implicate BRCA1 in the DNA damage response has come from the observation that BRCA1-deficient cells are hypersensitive to a variety of DNA damaging agents, including ionizing and ultraviolet radiation, and certain radiomimetic agents (*18*). A more specific function for BRCA1 in DNA damage repair was suggested by the observation that BRCA1-deficient cells exhibit overt defects in the repair of chromosomal double-strand breaks

by homologous and non-homologous recombination (*39,40*). Further studies documenting complex formation between BRCA1 and DNA repair proteins, including RAD50/MRE11/NBS1, RAD51, MSH2, MSH6, and MLH1 have provided additional evidence to suggest the direct participation of BRCA1 in the DNA repair process itself (*41-43*).

BRCA1 in Cell Cycle Checkpoint Control

DNA repair processes must be coordinated with control of cell cycle transit in order to ensure that damaged chromosomal DNA is repaired before it is replicated or segregated. There is considerable evidence to suggest that BRCA1 occupies a central and direct role in the activation of cell cycle checkpoints induced by DNA damage. First, BRCA1-mutant cells exhibit defects in DNA damage-induced S and G2/M cell cycle checkpoints (*38,44*). Second, BRCA1 is rapidly phosphorylated following DNA damage by cell cycle checkpoint kinases, suggesting that it may function downstream of DNA damage sensors that trigger cell cycle checkpoints (*45-47*). Finally, BRCA1 has been shown to regulate the expression of cell cycle checkpoint control genes, including *p21* and *GADD45* that function in G1/S and G2/M cell cycle checkpoints, respectively (*48,49*).

BRCA1 in Cell Growth and Differentiation

Evidence to suggest that BRCA1 occupies a fundamental role in the control of cell growth and differentiation comes from the observation that homozygous deletion of murine Brca1 results in early embryonic lethality accompanied by developmental retardation and cellular proliferation defects (*50*). This phenotype can be explained in part by the role of BRCA1 in the DNA damage response, since targeted deletions in *p53* or its downstream effector,

p21, can delay the early embryonic lethality associated with homozygous Brca1-deficiency (*50*). While the delayed embryonic lethality accompanying inactivation of *p53* has been ascribed to the accumulation of gross chromosomal defects incompatible with life, the possibility also exists that Brca1 is required for transit through a critical point later in the developing embryo.

Direct evidence to implicate BRCA1 in the regulation of cell growth and differentiation has come from analysis of transgenic mice carrying a *Brca1* allele that can be targeted for conditional inactivation specifically in the mammary gland. Mammary-specific inactivation of *Brca1* in female mice elicits defects in ductal morphogenesis and tumors associated with genetic instability, aneuploidy, and chromosomal rearrangements (*51*). Thus, in addition to independent support for BRCA1 as a breast tumor suppressor, this mouse model has also revealed a critical role for BRCA1 in mammary development.

BRCA1 ACTIVITIES

Genetic studies have thus revealed *BRCA1* to be an essential tumor suppressor with critical functions in the cellular DNA damage response and cell growth and differentiation. In parallel, biochemical and molecular biological analyses have been conducted in order to understand how BRCA1 executes these functions. These analyses have linked BRCA1 to a variety of activities through which it might possibly mediate its biological functions.

BRCA1 in Transcription Control

A role for BRCA1 in transcriptional regulation was initially indicated by the observation that its BRCT domain manifests an inherent transactivation function that is sensitive to cancer-predisposing mutations (*52*). Consistent with such activity, this region has been shown to

interact with the basal transcription machinery as well as a variety of transcriptional co-activators, including the histone acetyltransferase p300, and the catalytic subunit of the chromatin remodeling SWI/SNF complex, hBRG1 (*53-56*). Somewhat paradoxically, the BRCT domain also mediates the interaction of BRCA1 with transcriptional corepressors including the CtIP/CtBP complex and histone deacetylases (HDACs) (*57,58*). These observations have prompted speculation that BRCA1 may function as a context-dependent transcription factor, one whose ability to function as an activator or repressor is determined by its associated cofactors.

Consistent with possibility, recent studies utilizing gene expression profiling methodologies have revealed that ectopic overexpression of BRCA1 can induce or repress a diverse array of genes implicated in cell growth control, cell cycle regulation, apoptosis, and DNA replication and repair (*49,59,60*). Thus, by virtue of its transcriptional regulatory activity, BRCA1 could influence cellular responses downstream of DNA damage-induced signals, including DNA repair and cell cycle checkpoint activation.

Other Potential Activities of BRCA1

The mechanistic basis by which BRCA1 participates in transcription and DNA damage repair processes remains to be established. Most BRCA1 in the cell resides in stable complex with additional proteins, and one possibility is that BRCA1 functions as a molecular scaffold that facilitates the assembly of multiprotein machines responsible for DNA damage repair and transcription. Alternatively, by virtue of its association with chromatin remodeling activities, BRCA1 could variously promote or disrupt nucleosome-mediated condensation of DNA at gene promoters and/or DNA damage sites, thus precluding or facilitating access of repair and transcription factors, respectively. Finally, recent studies have identified a ubiquitin ligase

activity for BRCA1, thus raising the intriguing possibility that many of its pleiotropic activities could derive from the ability of BRCA1 to selectively mark proteins for destruction by the proteosome (*23,61-63*). The identification of physiological substrates of BRCA1-targeted ubiquitination will represent an important area of future investigation.

BRCA1 and TISSUE-SPECIFIC TUMOR SUSCEPTIBILITY

The DNA damage response pathways that converge on BRCA1 are likely universally conserved among different cell types, and BRCA1 is thus likely to function ubiquitously in the maintenance of genome integrity. Nonetheless, mutational inactivation of *BRCA1* leads principally to cancer of the female breast and ovary, and the underlying basis for its tissue- and gender-specific tumor suppressor properties remains poorly defined.

Several mutually compatible models have been proposed to explain how inactivation of BRCA1 could have restricted consequences in the breast and ovary. Because loss of heterozygosity at the *BRCA1* locus is a prerequisite for tumorigenesis, the frequency at which the second allele is lost in *BRCA1* mutation carriers could be higher in breast and ovarian, as opposed to other, epithelial cell populations (*64*). Alternatively, breast and ovarian epithelial cells might own a unique proclivity for protracted survival in the absence of BRCA1, thereby permitting the accrual of secondary mutations critical for tumorigenesis (*65*). On the other hand, recent data links BRCA1 to X chromosome heterochromatinization (*66*), suggesting that BRCA1 disruption could educe the overexpression of X-chromosome genes linked to breast and ovarian cancer. Finally, BRCA1 could fulfill a unique function in breast and ovary beyond its generally ubiquitous role as cellular caretaker, one whose disruption might promote tumorigenesis.

In this regard, BRCA1 has been shown to play a crucial role in the growth and

differentiation of the mammary gland (*30,51*). Furthermore, a considerable body of evidence supports a close relationship between mammary gland development and tumorigenesis. For example, it is well established that women who complete their first full-term pregnancy early in life carry a reduced lifetime risk of breast cancer (*67*). The underlying basis for this protective effect is believed to derive from estrogen-induced differentiation and consequent elimination of epithelial cell structures most susceptible to malignant transformation (*68*). Conceivably, mutational inactivation of BRCA1 could therefore perturb the normal program of mammary epithelial development and foster conditions compatible with tumorigenesis. This possibility is consistent with the observation that mammary-specific inactivation of BRCA1 in mice leads to defects in mammary gland development and tumor formation (*51*). However, the fact that mammary tumor formation in these mice is characterized by low frequency and long latency suggests the involvement of additional genetic alterations, possibly arising from genetic instability accompanying BRCA1 inactivation.

In this regard, the influence of steroid hormones, particularly estrogens, on the developing mammary gland is pertinent. The normal human mammary gland is comprised of a branching ductal system that develops under hormonal influences rudimentarily during puberty and only fully during pregnancy (*69*). In the normal breast, estrogen elicits mammary ductal growth during adolescence as well as lobuloalveolar proliferation during pregnancy (*70*). Because it is essential for the growth and proliferation of these epithelial cell structures, estrogen has been linked to the promotion and growth of breast cancer. Data from numerous studies suggest that estrogen can induce and promote breast cancer, while removal of the ovaries or administration of antiestrogens such as tamoxifen can oppose these effects (*71-73*). However, it is also known that estrogens and their metabolic byproducts can be mutagenic, suggesting that

estrogens may play an additional role in the initiation of tumor formation (*74*). Based on its dual role in breast cancer risk, two hypotheses invoking estrogen action have been proposed to explain the tissue-restricted tumor suppressor function of BRCA1. Importantly, these two models are not mutually exclusive and could suggest a combinatorial path to breast cancer, since they invoke BRCA1-mediated control at two distinct steps of tumorigenesis – initiation and progression (*75*).

According to one model, mutational inactivation of BRCA1 could render breast susceptible to the tissue-specific effects of estrogen-induced DNA damage. Consistent with this hypothesis is the observation that 4-hydroxyestradiol, a major oxidative metabolite of estrogen, is genotoxic (*74*). Thus, mutations in BRCA1 could compromise the response of breast epithelial cells to estrogen-induced DNA damage; this in turn could lead to genomic instability and a concomitant accrual of functionally inactivating mutations in other genes involved in breast tumorigenesis. In this way, BRCA1 mutations might enhance the probability of tumor formation arising from estrogen-induced DNA damage.

A second model to explain the tissue-specific tumor suppressor activity of BRCA1 invokes a role for BRCA1 in the modulation of estrogen signaling pathways and the control of cellular proliferation. The physiological effects of estrogen in the breast are mediated by cognate receptors that are expressed as two structurally related subtypes, estrogen receptor α (ERα) and β (ERβ) (*76*). These receptors are members of the nuclear receptor superfamily of ligand-activated transcription factors (*77*). Activation of ERα and ERβ by ligand binding elicits a conformational change in each receptor concomitant with dimerization and high-affinity binding to estrogen-response elements present within estrogen-responsive genes and through which the receptors promote high levels of transcriptional activation through targeted recruitment of co-

activators and the RNA polymerase II transcription machinery (*78,79*).

BRCA1 was first shown to influence estrogen signaling by Rosen and colleagues, who observed that BRCA1 was a potent inhibitor of the ligand-dependent transcriptional activity of ERα in cultured breast cancer cells (*80*). This repression was shown to be selective for the ligand-dependent transcriptional activation function (AF-2) within ERα. Subsequently, this group provided mechanistic insight into BRCA1 inhibition of ERα by showing that it occurs through a direct interaction between the N-terminus of BRCA1 and AF-2 within ERα (*81*). Importantly, tumor-associated mutations of BRCA1 compromised its ability to inhibit ERα activity (*81*).

These initial reports were followed by the observation that BRCA1 can also mediate ligand-independent transcriptional repression of ERα (*82*). In Brca1-null mouse embryo fibroblasts and BRCA1-deficient human ovarian cancer cells, ERα was observed to exhibit ligand-independent transcriptional activity that could not be observed in BRCA1-proficient cells. Furthermore, ectopic expression in Brca1-deficient cells of wild-type BRCA1, but not clinically validated BRCA1 missense mutants, restored ligand-independent repression of ERα in a manner dependent upon histone deacetylase activity. In human breast cancer cells, BRCA1 could be found in association with ERα on endogenous estrogen-responsive gene promoters before, but not after, estrogen stimulation. Finally, attenuation of BRCA1 expression in estrogen-dependent human ovarian cancer cells could be correlated with increases in both the estrogen-independent transcription of ERα-target genes and estrogen-independent cellular proliferation (*82,83*). Based on these observations, it was proposed that BRCA1 represents a ligand-reversible barrier to transcriptional activation by unliganded ERα. Coupled with previous studies by Rosen and colleagues, these findings suggest a possible mechanism by which functional inactivation of

BRCA1 could promote tumorigenesis through inappropriate hormonal regulation of mammary epithelial cell proliferation.

Several observations arising from these studies warrant further consideration. First, should BRCA1 function to inhibit the ligand-dependent transcriptional activity of ERα (*80,81*), it seems unlikely to do so through a mechanism that involves promoter-bound ERα, since chromatin immunoprecipitation analysis revealed the association of BRCA1 with ERα at endogenous estrogen-response elements prior to, but not following, estrogen stimulation (*82*). This suggests that BRCA1 may inhibit ERα activity through alternative mechanisms. Second, the fact that BRCA1-mediated repression of both liganded and unliganded ERα is similarly abrogated by clinically validated BRCA1 missense mutations suggests that its ERα repression function is important for the biological activity of BRCA1 in breast tumor suppression.

Recently, a functional interaction between BRCA1 and ERα has been implicated in angiogenesis. Specifically, BRCA1 was shown to inhibit ERα-mediated transcriptional activation of vascular endothelial growth factor (VEGF) gene transcription and protein secretion (*84*). The authors speculate that mutational inactivation of BRCA1 could promote tumor formation and angiogenesis through improper hormonal regulation of VEGF expression.

THE BRCA1/ER PARADOX

A function for BRCA1 in the modulation of estrogen signaling through inhibition of ERα activity could provide a basis to explain its linkage to breast cancer, which is an estrogen-dependent tumor type. However, it does not explain why BRCA1 mutations are linked to ovarian cancers, which are not primarily estrogen-dependent for growth. Nor does it explain why BRCA1 mutations are not implicated in the etiology of other estrogen-dependent tumor

types, such as endometrial and cervical cancers. A possible explanation for these paradoxical observations could derive from the tissue-specific expression of nuclear receptor co-regulators that conversely facilitate or antagonize the function of BRCA1 as an inhibitor of ERα activity. In this regard, the transcriptional coactivator p300 was recently implicated in modulation of BRCA1-mediated ERα repression (*85*). Furthermore, the ability of BRCA1 to repress ERα transcriptional activity correlated with its ability to down-regulate p300 levels in breast and prostate, but not cervical, cancer cells. Thus, BRCA1-mediated ERα repression may in part be dependent on tissue-specifically expressed cofactors.

It is well established that a significant proportion of BRCA1-associated breast tumors are negative for ERα expression (*12,86*), a clinical observation apparently incongruent with models invoking BRCA1 in the control of breast epithelial cell proliferation through modulation of ERα activity. A definitive explanation for this observation is precluded by a current lack of knowledge regarding how ERα-negative breast tumors arise. Several models have been proposed to explain the genesis of ERα-negative tumors, none of which precludes a possible link between BRCA1 and ERα as a factor in tumor development.

According to one model, ERα-negative tumors are hypothesized to arise from the loss of ERα expression during the clinical evolution of ERα-positive tumors (*87,88*). In this case, it is possible that the loss of ERα expression is a relatively late event in breast tumor progression, one that may have occurred after any proliferative advantages conferred upon transformation-initiated cells by homozygous BRCA1 mutation have ensued. Alternatively, it has been proposed that ERα-negative and ERα-positive tumors are distinct entities that reflect the receptor status of their clonal origins (*87,89*). In this regard, recent data suggest a model in which proliferation of ERα-negative cells is controlled by paracrine growth factors secreted from

ERα-positive cells in an estrogen-dependent manner (*90-94*). In this case, BRCA1 disruption could promote the release from ERα-positive cells of growth factors that stimulate the proliferation of ERα-negative tumors. Finally, the recent discovery of ERβ raises the possibility that this receptor mediates the proliferative response to estrogen in cells traditionally considered to be negative for ER expression (*95,96*). In this regard, it has recently been determined that ERβ is expressed during the immortalization and transformation of ERα-negative human breast epithelial cells (*95*). The functional role of ERβ-mediated estrogen signaling in the pathogenesis of breast cancer is currently unknown. Recently, it was reported that BRCA1 does not repress the ligand-dependent activity of ERβ (*16*). However, the possibility that BRCA1 modulates the ligand-independent activity of this estrogen receptor isoform has not yet been tested.

PERSPECTIVES

All available evidence to date suggests that endogenous exposure to female reproductive hormones is a principal determinant of breast cancer risk among *BRCA1* mutation carriers (*7*). Consequently, the suggestion that BRCA1 modulates estrogen signaling in the breast could have significant implications for the treatment of hereditary breast cancer with ER antagonists. In this regard, the use of tamoxifen as an adjuvant treatment for *BRCA1*-associated breast cancers has recently been evaluated in several large-scale retrospective clinical studies. Unfortunately, conflicting results have thus far been reported, thereby precluding a definitive assessment of the efficacy of tamoxifen in reducing hereditary breast cancer risk. An initial report from the Hereditary Breast Cancer Clinical Study Group (HBCCSG) that tamoxifen reduces the risk of contralateral breast cancer in BRCA1 mutation carriers (*97*) was followed by a report from the

U.S. Breast Cancer Prevention Trial (BCPT) that tamoxifen did not reduce breast cancer incidence among healthy *BRCA1* mutation carriers (*98*).

Several hypotheses have been proposed to explain the discordant results from these two studies. Because most *BRCA1*-associated breast cancers are ER-negative and tamoxifen is ineffective in the prevention and treatment of ER-negative breast cancers among women in the general population, a disproportionately high number of ER-positive *BRCA1*-associated breast cancers in the HBCCSG study could explain its contrasting results from those of BCPT (*99*). However, more recent data suggests that tamoxifen can significantly reduce the risk of breast cancer mortality in *BRCA1*-mutation carriers irrespective of ER status (*100*). These findings suggest that among ER-negative breast tumors, those arising in *BRCA1*-mutation carriers may respond differently to tamoxifen than those among women in the general population. Accordingly, earlier conclusions that tamoxifen has no role in the prevention or treatment of *BRCA1*-associated breast cancers may be premature.

A critical question regarding the potential prophylactic use of tamoxifen to reduce breast cancer incidence among *BRCA1*-mutation carriers concerns the appropriate age at which chemopreventive treatment should be initiated. Data from the BCPT analysis is not informative in this regard, since tamoxifen administration was initiated after age 35. Is it possible that tamoxifen could function prophylactically if administered at a younger age? Recent data from several clinical studies warrant consideration in this regard.

First, among *BRCA1*-mutation carriers, Rebbeck and colleagues reported that early (premenopausal) oophorectomy substantially reduces subsequent breast cancer risk, while Narod and colleagues observed that oophorectomy and tamoxifen independently reduce breast cancer risk to similar extents (*97,101*). Consequently, early treatment with tamoxifen might be expected

to reduce breast cancer incidence in *BRCA1*-mutation carriers. Second, recent work suggests that a principal risk factor for the development of hereditary breast cancer may be a heightened vulnerability of breast epithelium to the flood of hormones produced during puberty rather than a protracted exposure to ovarian hormones over the course of many years (*102*). Thus, for genetically predisposed individuals, tumorigenic potential might be realized through a pathological response to physiological signals early in breast development. If this is true, might disruption of BRCA1 elicit tumorigenesis by removing effective constraints on hormonal surges early in breast development? If so, effective chemoprevention in *BRCA1*-mutation carriers might require a means to reduce such vulnerability by antagonism of estrogen action for only a limited period but at a much earlier age than previously considered. Although purely speculative, this possibility is nonetheless consistent with established roles for BRCA1 in the control of breast development and the modulation of estrogen signaling.

As a decade approaches since its initial discovery, essential and universal functions for BRCA1 have been described. In the decade to come, we can anticipate that further disclosure of its biological activities will clarify the role of BRCA1 as a breast tumor suppressor and identify suitable inroads for future intervention in breast cancer.

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FIGURE LEGEND

Figure 1. Structural and functional motifs in BRCA1 and ERα are indicated above the schematic diagram of each protein. BRCA1 features named: RING domain, BRCT domain, NLS (nuclear localization signals), and exon 11-coding region. ERα features named: hormone-dependent activation function (AF-2), hormone-independent activation function (AF-1), DNA-binding domain (DBD), ligand-binding domain (LBD).

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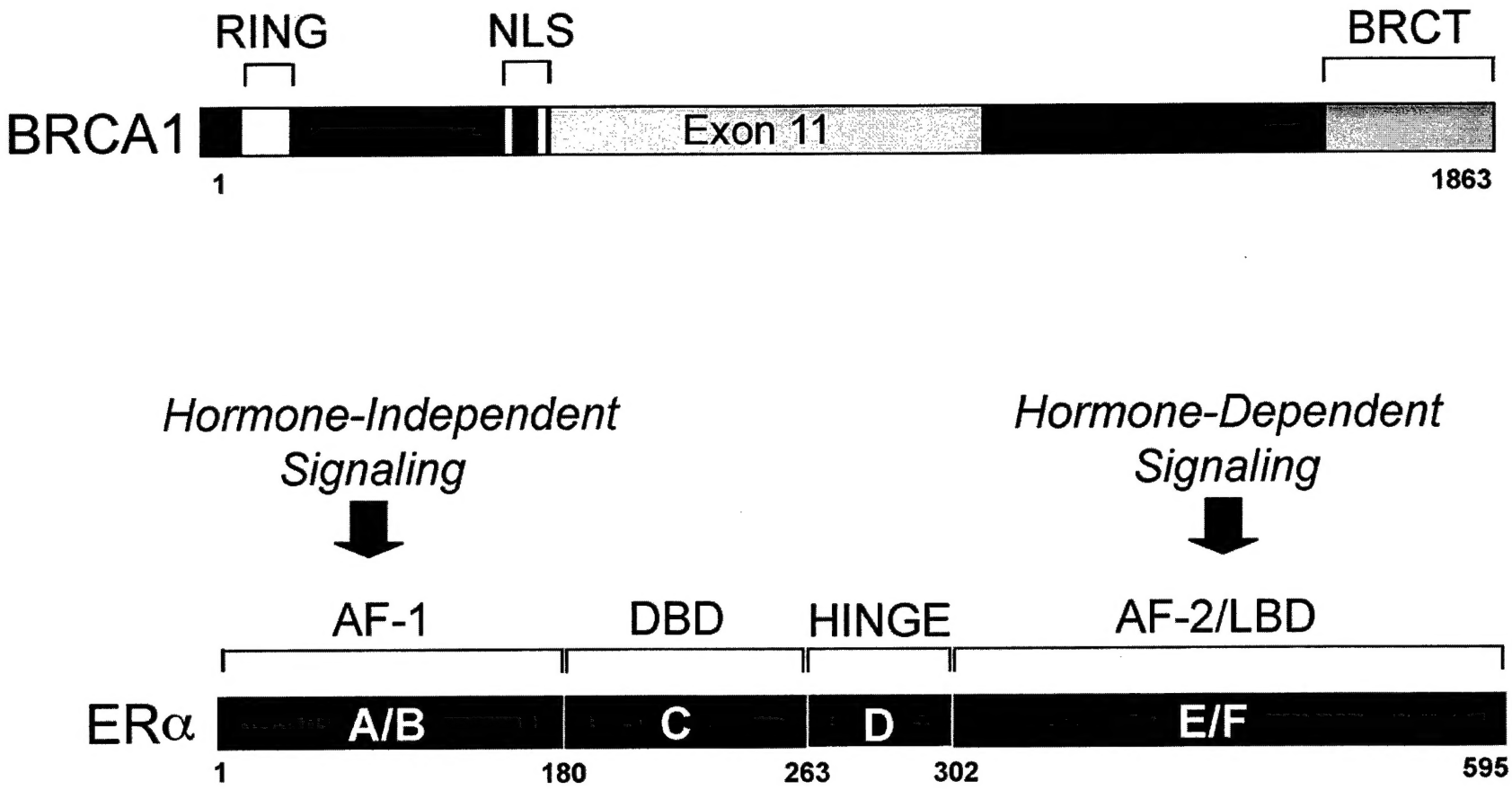
Figure 1. Structural and functional motifs in BRCA1 and ERα are indicated above the schematic diagram of each protein. BRCA1 features named: RING domain, BRCT domain, NLS (nuclear localization signals), and exon 11-coding region. ERα features named: hormone-dependent activation function (AF-2), hormone-independent activation function (AF-1), DNA-binding domain (DBD), ligand-binding domain (LBD).

Figure 1


RING
NLS
BRCT
BRCA1
Exon 11
1
1863
Hormone-Independent Signaling
Hormone-Dependent Signaling
AF-1
DBD
HINGE
AF-2/LBD
ERα
A/B
C
D
E/F
1
180
263
302
595